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# MEF2C, a MADS/MEF2-family transcription factor expressed in a laminar distribution in cerebral cortex

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**ABSTRACT** **We have cloned cDNA encoding a human transcription factor that belongs to the MEF2 (myocyte-specific enhancer-binding factor 2) subfamily of the MADS (MCM1–agamous–deficiens–serum response factor) gene family. This factor, which we have named MEF2C, binds specifically to the MEF2 element and activates transcription via this element. Specific isoforms of this factor are found exclusively in brain and are robustly expressed by neurons in cerebral cortex. *In situ* hybridization indicates that the factor is expressed preferentially in certain neuronal layers of cortex and that expression declines during postnatal development. The unusual pattern of expression in brain suggests that this transcription factor may be important in the development of cortical architecture.**

Mammalian cerebral cortex has a striking laminar structure. Neurons in different layers have specialized morphologies and distinctive functions. Cortical lamina develop in a well-defined temporal and spatial sequence (1). The development and subsequent maintenance of the laminar pattern are presumably regulated by transcription factors that control gene expression (2). Given the complexity of the brain, some of these factors may be distinct from the factors that determine non-neuronal cell lineages. However, the common electrically excitable phenotype shared by neurons and muscle cells suggests that some genes in these two cell types may be subject to regulation by similar or overlapping sets of transcription factors.

We now describe a human transcription factor that has several alternatively spliced isoforms and is a member of the MEF2 (myocyte-specific enhancer-binding factor 2) subfamily of the MADS (MCM1–agamous–deficiens–serum response factor) gene family (3–8). Proteins in the MEF2 subfamily interact with the MEF2 DNA element, a regulatory sequence that has thus far been found to be functionally important in a variety of muscle-specific genes and possibly in the brain creatine kinase gene (8–11). Until now, however, no identified brain protein has been demonstrated to activate transcription through the MEF2 element. The MEF2 subfamily includes previously reported proteins derived from two human genes, MEF2 and xMEF2, each with alternatively spliced isoforms (7, 8). We have now cloned products of a third human gene, which is expressed at high levels in muscle and in cerebrocortical neurons and which we have named human MEF2C (hMEF2C).** Specific isoforms are expressed only in the brain, and we have cloned other isoforms from skeletal muscle cDNA libraries.

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## MATERIALS AND METHODS

**Library Screening.** A cDNA clone corresponding to amino acids 140–238 of hMEF2C was fortuitously identified when a human fetal brain cDNA library (12) was screened with monoclonal antibody HOPC8 (13) as part of an effort to identify cDNA clones expressed in the brain. The clone obtained initially contained an incomplete open reading frame and was used to rescreen the same library; several longer clones also containing incomplete open reading frames were identified. Because Northern blotting using one of these clones as probe revealed hybridization to bands in brain and skeletal muscle, we screened human fetal brain and muscle cDNA libraries (generously provided by L. M. Kunkel, Children's Hospital, Boston) to obtain additional clones. Both strands of the entire sequence were determined from at least one clone by the dideoxy method. Sequence analysis was performed with programs from the Genetics Computer Group package (14).

**Electrophoretic Mobility-Shift Analysis.** When *in vitro* translated proteins were used in the gel shift assays, 1.5 μl of reticulocyte lysate was incubated with 0.25 ng of $^{32}$P-labeled double-stranded oligonucleotide probe containing the MEF2 consensus sequence (8, 11), with 0.45 μg of poly(dI-dC) and 100 ng of a single-stranded oligonucleotide as competitors of nonspecific binding and with 100-fold excess of unlabeled double-stranded oligonucleotides as indicated. When nuclear extracts were used, the incubation mixture contained ≈5 μg of nuclear extract, protein, 0.25 ng of probe, and 3 μg of poly(dI-dC), and 100 ng of single-stranded oligonucleotide as nonspecific DNA competitors. For assays with antisera, 1 μl of serum was preincubated with the nuclear extract or *in vitro* translated protein for 15 min at room temperature (15).

**Fusion Protein and Antiserum Production.** A cDNA coding for amino acids 140–238 was subcloned into the pATH11 plasmid, and a TrpE fusion protein was isolated and used to immunize a rabbit, as described previously (16).

**Transfections.** The coding regions of hMEF2C and hMEF2C/Δ32 cDNA were subcloned into the mammalian expression vector pMT2 (17). Tissue culture and transient transfections were performed as described (18, 19). Five micrograms of the cDNA plasmid, 10 μg of the reporter construct containing a chloramphenicol acetyltransferase (CAT) gene, and 3 μg of pSV-βgal were cotransfected by calcium phosphate coprecipitation into HeLa cells. The cells

Abbreviations: MADS, MCM1–agamous–deficiens–serum response factor; MEF2, myocyte-specific enhancer-binding factor 2; hMEF2C, human MEF2C; CAT, chloramphenicol acetyltransferase; P*n*, postnatal day *n*.

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**The cDNA sequence of hMEF2C has been deposited in the GenBank data base (accession no. L08895).

were subjected to a glycerol shock after 18 hr and then harvested after an additional 48 hr. CAT activity was normalized for transfection efficiency by correcting for β-galactosidase activity from the cotransfected pSV-βgal plasmid (8, 19).

**Northern Blotting.** Total RNA was prepared as described (20), electrophoresed in formaldehyde/agarose gels, and transferred to Nytran (Schleicher & Schuell). Blots were probed with $^{32}$P-labeled random-primed probes.

***In Situ* Hybridization.** *In situ* hybridization was performed (21) on fresh-frozen 12-μm sections of rat brain. $^{35}$S-labeled RNA probes were generated by using a subclone containing cDNA corresponding to amino acids 140–238 of hMEF2C in the pGEM-3 vector. Essentially identical results were obtained using RNA probes derived from a subclone containing the last 96 bases of the open reading frame of hMEF2C and 1.3 kb of the 3′ untranslated region (data not shown).

## RESULTS AND DISCUSSION

**Sequence of hMEF2C.** We isolated multiple human cDNA clones that appear to represent alternatively spliced isoforms derived from a previously unrecognized gene belonging to the MEF2 subfamily (Fig. 1). Data base searches with the BLAST program (22) indicate that our clones have highly significant homologies with all members of the MADS family in the so-called MADS domain (4), which extends from the N terminus to amino acid 57 of our clones. Outside of the MADS domain, the sequence of our clones diverges from that of the other known members of the MADS family except for proteins in the MEF2 subfamily (7, 8). The MEF2 domain is adjacent to the MADS domain and is shared by hMEF2C and all other members of the MEF2 subfamily (Fig. 1 and ref. 8). The MADS and MEF2 domains are both necessary for specific DNA binding by members of the MEF2 subfamily (7, 8).

The deduced amino acid sequence of hMEF2C is most homologous to that of aMEF2, an isoform of the MEF2 gene (7, 8). The amino acid sequence of hMEF2C is identical to that of aMEF2 at 56 of the 57 amino acids of the MADS domain and at the next 39 amino acids, which include the MEF2 domain, with 80% homology at the nucleic acid level over the region coding for these 96 amino acids. The sequences of hMEF2C and aMEF2 are 58% identical at the amino acid level over the remainder of the sequence (Fig. 1). However, only hMEF2C exhibits a restricted nature of expression at the RNA level, with transcripts of some isoforms being limited exclusively to the brain (see below). Other known transcription factors in the MEF2 subfamily are more widely distributed at the RNA level, although at the protein level, their expression is tissue-specific and thus is presumably regulated by posttranscriptional mechanisms (7, 8).

Some of our clones from brain lack a 32-amino acid sequence (amino acids 368–399); we have designated this variant as hMEF2C/Δ32. These amino acids are also missing from some of the muscle clones. All of the muscle clones also lack an 8-amino acid sequence (amino acids 271–278); reverse transcriptase–polymerase chain reaction studies have indicated that isoforms containing these 8 amino acids are expressed exclusively in brain (data not shown). Interestingly, there is a gap in the homologous regions of some isoforms of the MEF2 gene, but not in the aMEF2 sequence (Fig. 1). Aside from these 8 amino acids, the coding regions of the muscle isoforms of hMEF2C are the same as those of the brain isoforms; the properties of the muscle isoforms will be described in greater detail in a separate report.

**Binding of hMEF2C to DNA.** The extent of the homology with members of the MEF2 subfamily suggested that the proteins expressed by our clones might also specifically bind to MEF2 binding sites. Fig. 2*A* shows that proteins encoded by hMEF2C and hMEF2C/Δ32 indeed bind to MEF2 targets. The pattern of binding to several double-stranded oligonucleotides with variant sequences was exactly that expected based on previous work with *in vitro* translated MEF2 subfamily members and with nuclear extracts from muscle (8, 11). This confirms specific binding of hMEF2C and hMEF2C/Δ32 to MEF2 elements. In addition, a double-stranded oligonucleotide based on the variant of the MEF2 sequence found in the brain creatine kinase gene (9, 10) competed with the MEF2 consensus probe (Fig. 2*A*), so our clones can also bind to this element. Our clones, however, do not appear to interact with an oligonucleotide containing a CArG box (Fig. 2*A*), a motif which binds other members of the MADS family, such as serum response factor (3), but not members of the MEF2 subfamily (7, 8).

To identify *in vivo* DNA-binding proteins corresponding to hMEF2C, we used a rabbit antiserum raised against a fusion protein containing amino acids 140–238 of hMEF2C. As shown in Fig. 2*B*, this antiserum specifically supershifts the MEF2 binding activity translated *in vitro* from hMEF2C cDNA clones but not from aMEF2 (8). Gel shift assays with nuclear extracts from fetal human cerebral cortex and rat brain demonstrated several MEF2 binding factors with different mobilities, but in each case only one band was shifted with the antiserum. This band had a mobility very similar to that of our *in vitro* translated proteins (Fig. 2*C*). This finding suggests that the factor encoded by the cDNA that we have cloned or a very similar one is present in both human fetal

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                  ----------MADS Domain----------  =========MEF2 Domain=========
hMEF2C:  1 MGRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCEIALIIFNSTNKLFQYASTDMDKVLLKYTEYNEPHESRTNSDIVETLRKKGLNGCDSPD 100
aMEF2:   1 MGRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCEIALIIFNSSNKLFQYASTDMDKVLLKYTEYNEPHESRTNSDIVETLRKKGLNGCES-- 98

       101 PDADDSVGHSPESEDKYRKINEDIDLMISRQRLCAVPPPNFEMPVSIPVSSHNSLVYSNPVSSLGNPNL----------LPLAHPSLQRNSMSPGVTHR 189
        99 PDADDYFEHSPLSEDRFSKLNEDSDFIFKRGP.PGLPPQNFSMSVTVPVTSPNALSYTNPGSSLVSPSLAASSTLTDSSMLSPPQTTLHRN.VSPGAPQR 196

       190 PPSAGNTGGLMG-GDLT--SGAGTSA-GNGYGNPRNSPGLLVSPG--NLNKNMQAKSPPPM---NLGMNNRKPDLRVLIPPGSKNTMPSVSEDVDLLLN. 279
       197 PPSTGNAGGMLSTTDLTVPNGAGSSPVGNGFVNSRASPNLIGATGANSLGKVMPTKSPPPPGGGNLGMNSRKPDLRVVIPPSSKGMMPPLSEEEELELNT 296

       280 QRINNSQSAQSLATPVVSVATPTLPGQGMGGYPSAISTTYGTEYSLSSADLSSLSGFNTASALHLGSVTGWQQQHLHNMPPSALSQLGACTSTHLSQSSN 379
       297 QRISSSQATQPLATPVVSVTTPSLPPQGLVY--SAMPTAYNTDYSLTSADLSALQGFNSPGMLSLGQVSAWQQHHL---GQAALSSLVA--GGQLSQGSN 389

       380 LSLPSTQSLNIKSEPVSPPRDRTTTPSRYPQ----------HTRHEAGRSPVDSLSSCSSSYDGSDREDHRNEFHSPIGLTRPS-PD 455
       390 LSINTNQNISIKSEPISPPRDR.MTPSGFQQQQQQQQQQQPPPPQPQPQPPQPQPRQEMGRSPVDSLSSSSSSYDGSDREDPRGDFHSPIVLGRPPNTE 488

       456 ERESPSVKRMRLSEGWAT 473
       489 DRESPSVKRMRM-DAWVT 505

                                  | Identical amino acid
                                  : Highly conservative substitution
                                  . Conservative substitution
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FIG. 1. Predicted amino acid sequence of hMEF2C aligned with that of aMEF2 (8), which is an isoform of RSRFC9 (7). The underlined amino acids are missing from some alternatively spliced isoforms as detailed in the text.

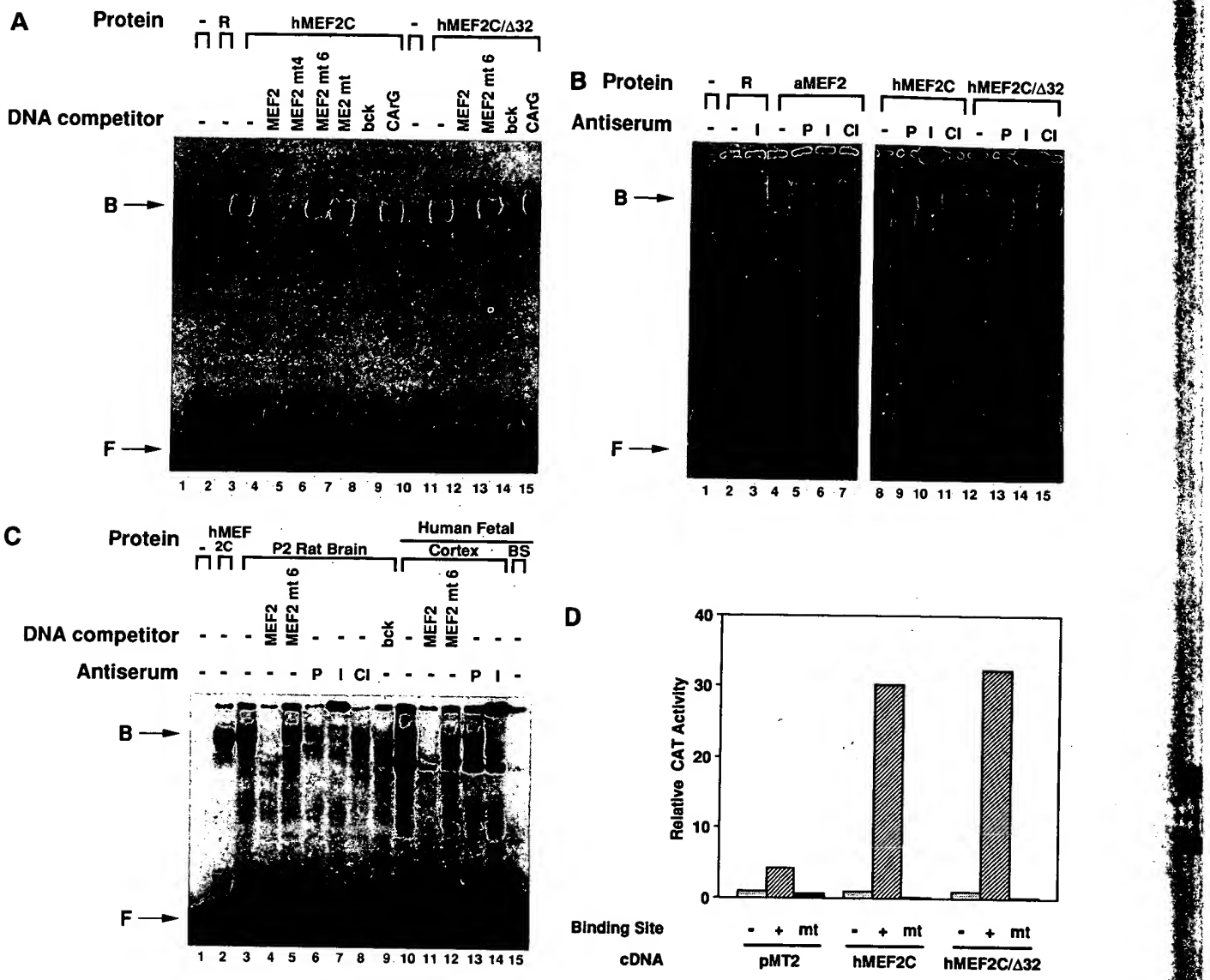


FIG. 2. Interactions of hMEF2C with the MEF2 element. (*A–C*) Electrophoretic mobility-shift assays. The probe was a double-stranded $^{32}$P-labeled oligonucleotide containing the MEF2 consensus binding site (8, 11), and the positions of free probe (F) and of bound probe (B) to hMEF2C are indicated by arrows. The double-stranded DNA oligonucleotide competitors—MEF2 mutant (mt) 4, mt 6, and mt, and CArG—are the same as used previously (8, 11). The brain creatine kinase (bck) oligonucleotide contained the core sequence 5′-ATGGGCTATAAAT-AGCCGCCA-3′ (9, 10). (*A*) Lanes 1 and 10 show free probe without any reticulocyte lysate, and lane 2 shows probe incubated with an unprogrammed reticulocyte lysate (R). (*B*) The binding specificity of the rabbit antiserum raised against amino acids 140–238 of hMEF2C is shown. Unprogrammed lysates (R) or lysates programmed with RNA transcribed from the indicated cDNA clones were preincubated with the immune (I) antiserum, with preimmune serum (P) from the same rabbit, or with a control immune (CI) rabbit antiserum against glial fibrillary acidic protein. The dark band at the top of lanes 10 and 14 is the band shifted by the immune antiserum. Note that the weak band near the top of lane 6 is also present when the reticulocyte lysate is preincubated with the immune antiserum (lane 3). (*C*) Activity of hMEF2C in brain extracts. Nuclear extracts from postnatal day 2 (P2) rat brain and from 20-week-old human fetal cerebral cortex or brainstem (BS) (18) were incubated with the $^{32}$P-labeled MEF2 consensus binding-site probe and with double-stranded oligonucleotide competitors or antiserum as indicated. The dark band at the top of lanes 7 and 14 is the band shifted by the immune antiserum. Only one of several bands that bind to the MEF2 probe is shifted; the other bands may represent other members of the MEF2 subfamily. *In vitro* translated hMEF2C incubated with the MEF2 probe is shown in lane 2; lane 1 contained free probe only. (*D*) Transcriptional activity of pE102CAT reporter constructs. Relative CAT activity is defined as the ratio of CAT activity for cotransfection of a given cDNA with the indicated pE102CAT reporter plasmid to CAT activity for cotransfection with pE102CAT itself (8). Reporter plasmids contained two copies of the MEF2 consensus binding site (+), two copies of the mutant oligonucleotide MEF2 mt (mt), or no inserted oligonucleotide (−). The relative activity when no oligonucleotide is inserted is 1 by definition and is shown only for comparison. Representative values from one determination of a total of two to four cotransfections are shown.

cerebral cortex and rat brain. In contrast, we found no MEF2 binding activity in human fetal brainstem (Fig. 2*C*). In control experiments to test the quality of the brainstem extract, we detected similar amounts of octamer-binding activity (23) in human fetal cerebral cortex and brainstem.

**Transcriptional Activation by hMEF2C.** In view of the sequence homology of our clones with other MEF2 proteins and the finding that hMEF2C and hMEF2C/Δ32 recognize the MEF2 element, we sought to determine whether hMEF2C and hMEF2C/Δ32 could activate transcription upon binding to the MEF2 element. Forced expression of hMEF2C and hMEF2C/Δ32 markedly enhanced transcription in HeLa cells cotransfected with a reporter gene containing two copies of the MEF2 element inserted into the embryonic myosin heavy chain promoter (Fig. 2*D*). In addition, transcription was enhanced in cotransfection experiments using a reporter (8) containing two copies of the MEF2 element inserted into a reporter gene containing the thymi-

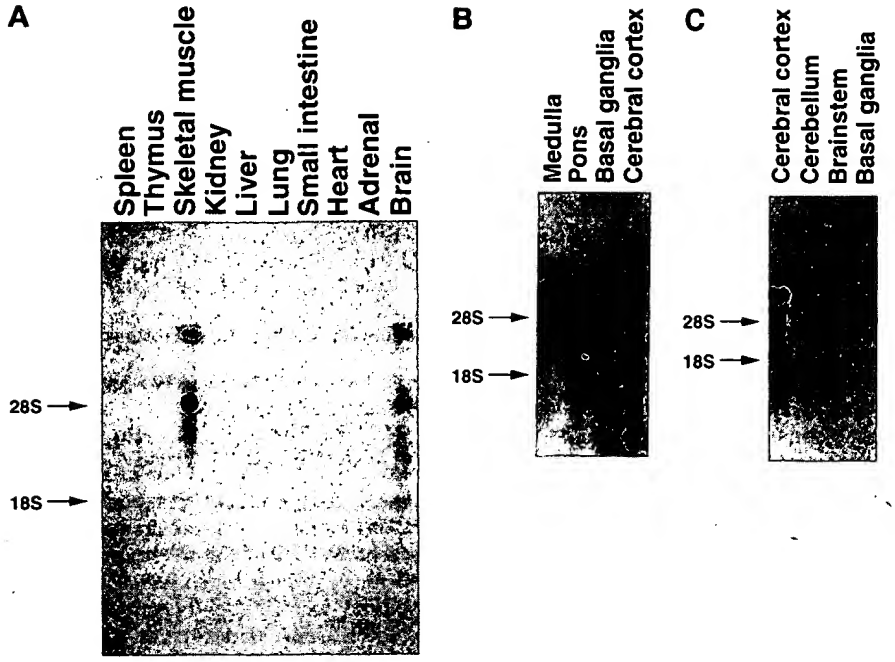


FIG. 3. Northern blot analysis of hMEF2C expression. Twenty micrograms of total RNA from the indicated tissues from a 20- to 22-week-old human fetus (*A*) and 10 μg of total RNA from the indicated regions of brain from a 20-week human fetus (*B*) and from a P8 rat (*C*) were probed with random-primed probes derived from a 1.4-kb cDNA restriction fragment containing a short stretch of 5′ untranslated cDNA and all but the last 91 bases of the open reading frame of hMEF2C. Equivalency of RNA loading was confirmed by stripping the blots and reprobing with multiple independent probes (*A*) and with a glyceraldehyde-3-phosphate dehydrogenase probe (*B* and *C*). The blots in *B* and *C* were also rehybridized with a probe derived from cDNA containing the last 96 bases of the open reading frame and 1.3 kb of the 3′ untranslated region, and the same bands were again visualized.

dine kinase promoter (data not shown). Thus, the proteins encoded by the hMEF2C and hMEF2C/Δ32 cDNAs activate transcription via the MEF2 element in the context of different promoters in HeLa cells. In addition, the two brain isoforms of hMEF2C transactivate to similar extents, and so the alternatively spliced domain that is missing from hMEF2C/Δ32 is not essential for transcriptional activation in this assay.

**Distribution of hMEF2C RNA Expression.** We also explored the expression of hMEF2C mRNA by Northern blot analysis. We detected transcripts of approximately 5 and 8 kb in human fetal brain and skeletal muscle, but not in a variety of other tissues (Fig. 3*A*). Northern blots of RNA from different regions of human fetal brain revealed that there were high levels of expression in cerebral cortex, with minimal levels in other brain regions (Fig. 3*B*). The low levels of expression detected in the pons and medulla by Northern blotting appear to contrast with the absence of MEF2 binding activity in brainstem extracts (Fig. 2*C*). This apparent dis-

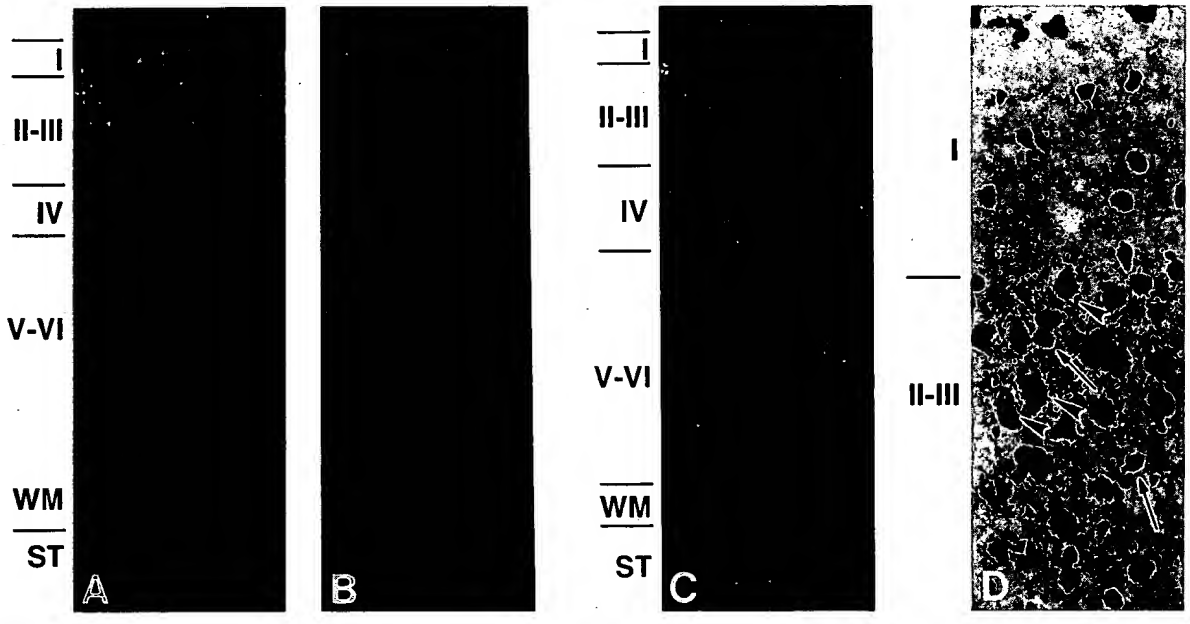


FIG. 4. *In situ* hybridization analysis of hMEF2C expression. (*A–C*) Dark-field photomicrographs of coronal sections of P8 (*A* and *B*) and P28 (*C*) rat brain probed with antisense (*A* and *C*)- and sense (*B*)-strand hMEF2C probes. Preferential labeling of neurons in layers II–IV is seen at P8 with the antisense probe (*A*), but no labeling is detectable with the sense-strand probe (*B*); at P28 with the antisense probe (*C*), there is a similar pattern of labeling of neurons predominantly in layers II–IV, but the signal is less intense, and the distinction between layers II–IV and V–VI is less striking. The approximate boundaries of neocortical layers I, II–III, IV, and V–VI, and of white matter (WM) and striatum (ST) were determined in *A–C* by examination of cresyl violet counterstaining under brightfield optics. Note that the laminar pattern is essentially the same in *B* as in *A*, which is a neighboring section. (×23.) (*D*) A high-power brightfield photomicrograph of P8 rat cortex is shown counterstained with cresyl violet. Silver grains are localized over neuronal cell bodies (e.g., indicated by arrowheads) in layers II–III, but not over non-neuronal cells in layer I or over cells with small nuclei that appear to be non-neuronal in layers II–III (e.g., indicated by arrows). High-power examination of the sections also revealed that there appeared to be more silver grains per labeled neuron in layers II–IV than in layers V–VI (not shown at high power); thus, the increased signal in layers II–IV is not merely an effect of increased density of cells. (×210.)

crepancy, however, may reflect disparate sensitivity of the two techniques or regulation at a posttranscriptional level, as is the case for other members of the MEF2 subfamily (8). Similar results were obtained for Northern blots of rat brain, in which abundant transcripts of similar sizes to the human bands were identified in RNA from cerebral cortex, while only low levels of transcripts were found in RNA from other brain regions (Fig. 3*C*). Taken together with the gel shift experiments (Fig. 2*C*), these results constitute strong evidence that hMEF2C is expressed preferentially in human cerebral cortex and that a closely related homologue is expressed in rat cerebral cortex. Northern blots also demonstrated that expression declined with age in rats from postnatal day 2 to adulthood (data not shown).

*In situ* hybridization on sections of rat brain at 5 postnatal ages (P2, P8, P15, P28, and adult) yielded striking results (Fig. 4). Expression was limited to neurons and was consistently higher in the outer layers of neurons in neocortex (layers II–IV) than in the infragranular layers (layers V–VI). Expression was higher in infragranular neurons, however, than in striatal neurons. In contrast, no expression was detected in the non-neuronal cells of layer I. In addition, expression was greater in younger postnatal animals than in older ones; finally, preliminary studies of embryos indicate that hMEF2C is not expressed in proliferating precursor cells in the ventricular zone.

The regional, laminar, and developmental specificity of hMEF2C expression suggests that differential expression of this transcription factor may accompany and, perhaps in part, direct neuronal differentiation and maturation, especially in the process of cortical lamination. In this regard, members of the MADS family, such as the agamous protein, have roles in morphogenesis in flowering plants (4–6), and other members of the MEF2 subfamily are likely to be critical in muscle cell differentiation (8). In addition, homeodomain transcription factors of the POU class have been found to have a laminar distribution in cerebral cortex (2), and homeodomain proteins can interact cooperatively with members of the MADS family (24, 25). Control of cortical lamination may therefore involve an interaction between the proteins encoded by the cDNAs we have identified and homeodomain proteins.

In conclusion, we have identified and characterized hMEF2C, a member of the MADS family that has several isoforms, specifically binds to the MEF2 element and transactivates reporter constructs via this element, and is preferentially expressed at high levels by specific neurons in the cerebral cortex. Moreover, the laminar pattern of expression of hMEF2C in neocortex suggests that it may have a role in the laminar differentiation of cortical neurons.

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